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Sequence Analysis and Evolutionary Perspectives of ROB-1 β-Lactamase

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The nucleotide sequence of the ROB-1 β-lactamase gene from *Haemophilus influenzae* plasmid R_{Rob} was determined. The structural gene encodes a polypeptide of 305 amino acids, with an estimated molecular mass of 30,424 for the mature form of the protein. The ROB-1 gene showed low homologies with other β-lactamases at the nucleic acid level. By using two statistical computer methods, assessment of the extent of similarity between ROB-1 and other known β-lactamase amino acid sequences suggested that ROB-1 is a class A enzyme. Alignment of class A β-lactamases with ROB-1 identified conserved residues. The use of a mutation matrix for detecting distance relationships indicated that ROB-1 has higher values and homologies with β-lactamases of gram-positive bacteria, giving insight into its ancestry and divergence.

The ROB-1 β-lactamase was first identified in ampicillin-resistant *Haemophilus influenzae* type b isolates by Rubin et al. (31) in 1981. The following year, plasmid-mediated ampicillin resistance was reported in *Actinobacillus pleuropneumoniae* (17), and patterns of resistance suggested a TEM-type β-lactamase. Medeiros et al. (25) discovered that plasmid-mediated β-lactamases from *H. influenzae* and *A. pleuropneumoniae* were indistinguishable by isoelectric focusing or DNA hybridization. Further studies confirmed that the ROB-1 structural gene showed no cross-hybridization with other *bla* genes (22). The same enzyme was also found on plasmids and on the chromosome in *Pasteurella* strains (23). A prevalence study of the ROB-1 β-lactamase among 161 ampicillin-resistant *H. influenzae* strains in the United States showed that 8% produced ROB-1, whereas 92% of strains produced TEM-1 (12).

Based on the broad-spectrum nature of the ROB-1 enzyme that hydrolyzes penicillins and cephalosporins (25), ROB-1 was identified as a class 2b enzyme in the classification scheme of Richmond and Sykes (29), as recently modified by Bush (7). Considering the biochemical nature of ROB-1, it would be tempting to speculate that this enzyme is a typical class A β-lactamase in the classification of Ambler (1) and, presumably, is related to TEM-type enzymes.

Here we report the nucleotide sequence of the ROB-1 structural *bla* gene isolated from *H. influenzae* plasmid R_{Rob} and compare it with other known β-lactamases to gain insight into its evolution.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* DH5α was the recipient strain for recombinant plasmids pMON418 and pMON419. JM101, the recipient for M13mp19 phage, was conserved on minimal medium without proline (40). *E. coli* HB101 was the recipient host for other plasmids. In all cases, bacterial cells were grown on tryptic soy agar (Difco Laboratories, Detroit, Mich.) containing appropriate antibiotics (ampicillin, 20 μg/ml; kanamycin, 50 μg/ml). For pBGS18$^+$ and pBGS19$^+$ and pTZ18R phagemids, production of single-stranded DNA with the helper phage M13K07 was done as described by Spratt et al. (35) and Vieira and Messing (38).

Preparation of DNA and related techniques. Plasmids were prepared by the cleared lysate method, with modification to cell lysis (1 mg of lysosyme per ml, 0.075 M disodium EDTA, 1% sodium dodecyl sulfate), and purified by cesium chloride-ethidium bromide gradient ultracentrifugation (33). Plasmid DNA was digested with restriction enzymes by using the conditions recommended by the manufacturers (*Bam*HI, *Dra*I, and *Alu*I [Bethesda Research Laboratories Inc., Gaithersburg, Md.]; *Eco*RI, *Eco*RV, and *Hin*dIII [Pharmacia LKB Biotechnology, Baie d'Urfée, Quebec, Quebec, Canada]). Restricted DNA fragments were separated by agarose gel (0.8%) electrophoresis. Subcloning and transformation were done as described by Silhavy et al. (33).

Nucleotide sequencing and computer analysis. DNA sequencing was performed by the dideoxy polymerase chain termination method (32). DNA fragments cloned in M13mp19 were sequenced with the Klenow fragment (International Biotech. Inc., Toronto, Ontario, Canada), and clones in pTZ18R or pBGS18$^+$ and pBGS19$^+$ were sequenced by using the phage T7 polymerase (37) (Pharmacia). In addition to the universal primers (Pharmacia), we synthesized four 17-mer oligonucleotide primers by phosphite triester chemistry (3) on the Gene Assembler Plus apparatus (Pharmacia). Oligonucleotides were purified on 20% polyacrylamide-urea gels (3). Analysis of the DNA sequence was performed with the software package of the University of Wisconsin Genetics Computer Group (15) and the protein sequence analysis software of the Protein Identification Resource (13). Scores obtained with the ALIGN program of Dayhoff (13) by using the mutation matrix are based on amino acid replacements between two aligned proteins indicating distance relationships. Searches for similarities of ROB-1 with other DNA sequences and proteins were performed with the GenBank, European Molecular Biology Laboratory, and National Biomedical Research Foundation data bases.

RESULTS

Physical mapping and subcloning of the ROB-1 structural gene. The recombinant plasmid pMON401 (Ap^r Cm^r) was constructed from a partial *Sau*3A digest of R_{Rob} (Ap^r) cloned into the *Bam*HI site of pACYC184 (22). In contrast to an

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TABLE 1. Bacterial strains, plasmids, and phages used in this study

Strain, plasmid, or phage	Relevant characteristics[a]	Reference or source
E. coli strains		
HB101	F⁻ *hsdS20*(r_B^-, m_B^-) *supE44 ara-14 galK-2 lacY1 proA2 leu endA thi-2 rpsL20*(Str^r) *xyl-5 mtl-1* RecA13	6
JM101	SupE *thi* Δ(lac-proAB) (F′ *traD36 proAB lacI^{q}ZΔM15*)	40
DH5α	F⁻ ϕ80d*lacZ*ΔM15 Δ(*lacZYA-argF*) U169 *recAI endAI hsdR17* (r_k^-, m_k^+) *supE44 thiI gyrA relAI*	16
Plasmids or phages		
pACYC184	Cm^r Tc^r	11
pBGS18$^+$/19$^+$	Km^r *lacPOZ′*	35
R_{Rob}	Ap^r	31
pTZ18R	Ap^r *lacPOZ′*	38
pVM105	Ap^r Su^r	17
M13mp19	*lacPOZ′*	26
M13K07	Km^r	38
pMON401	Cm^r Ap^r	This work
pMON402	Ap^r	This work
pMON418	Km^r Ap^r	This work
pMON419	Km^r Ap^r	This work

[a] Abbreviations: Ap, ampicillin; Cm, chloramphenicol, Km, kanamycin; Su, sulfonamide; Tc, tetracycline.

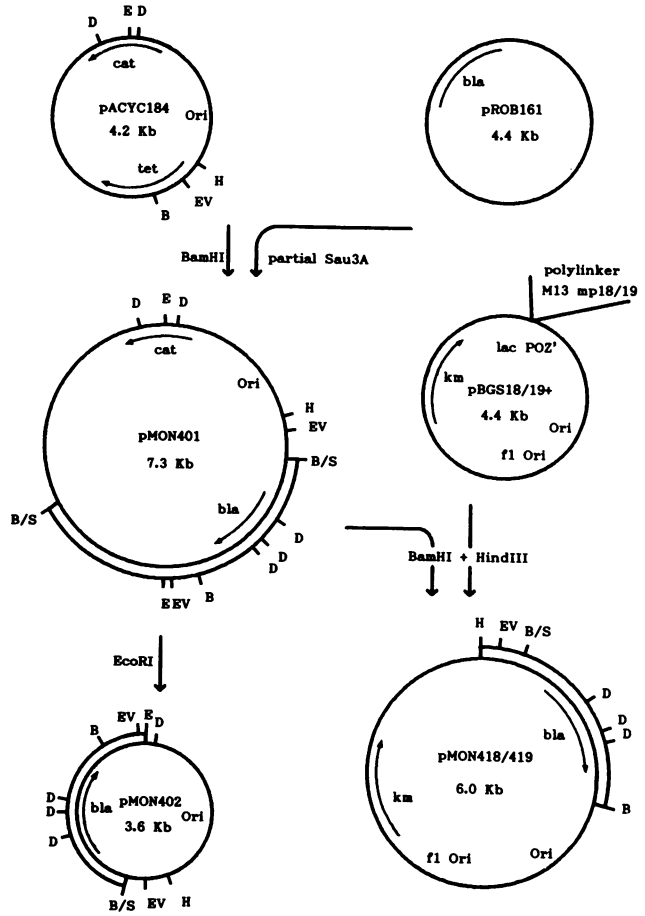

FIG. 1. Isolation of the ROB-1 *bla* gene. Plasmid pMON401 was constructed by cloning R_{Rob} *Sau*3A fragments into the *Bam*HI site of pACYC184. The *Eco*RI deletion plasmid pMON402 localized the *bla* gene between the *Eco*RI and the *Bam*HI-*Sau*3A sites, while *Bam*HI-*Hin*dIII subcloning into pBGS18$^+$/19$^+$ gave pMON418 and pMON419. Abbreviations: bla, β-lactamase gene; cat, chloramphenicol acetyltransferase gene; f1 Ori, f1 phage replication origin; km, kanamycin resistance; Ori, replication origin; tet, tetracycline resistance. Restriction sites are abbreviated as follows: B, *Bam*HI; B/S, *Bam*HI-*Sau*3A; D, *Dra*I; E, *Eco*RI; EV, *Eco*RV; H, *Hin*dIII.

earlier report (22), finer restriction mapping confirmed that the *Bam*HI site found in pMON401 is not at one extremity of the insert but is internal to the cloned fragment. To localize the ROB-1 *bla* structural gene precisely, we mapped pMON401 with restriction endonucleases *Bam*HI, *Dra*I, *Eco*RI, and *Eco*RV (Fig. 1). Construction of pMON402 eliminated a 3.7-kilobase (kb) *Eco*RI fragment with retention of ampicillin resistance, thus localizing the ROB-1 *bla* gene on a 1.5-kb *Eco*RI and *Bam*HI-*Sau*3A fragment of the pMON401 map. Directional cloning of a 1.8-kb *Bam*HI-*Hin*dIII fragment into pBGS18$^+$ and into pBGS19$^+$ in both orientations (pMON418 and pMON419) localized the structural gene in a small region sufficient for nucleotide sequencing and suggested that ROB-1 is expressed from its own promoter.

Nucleotide sequence of the ROB-1 gene. The nucleotide sequencing strategy of the ROB-1 gene is illustrated in Fig. 2. First, a 310-base-pair (bp) *Eco*RI-*Bam*HI fragment and a 242-bp *Alu*I fragment cloned in M13mp19 were sequenced. By cloning a 964-bp *Dra*I-*Hin*dIII fragment into pTZ18R, we determined the sequence of 454 bp. Finally, by using these sequences, we synthesized a series of oligonucleotide primers (Fig. 2) and completed the nucleotide sequences for the 1,775-bp *Bam*HI–*Sau*3A-*Eco*RI fragment (GenBank accession number, M33576). The nucleotide sequence region encompassing the ROB-1 structural gene was determined for both strands.

Analysis of the entire sequence for coding regions showed an open reading frame encoding a putative protein of 305 amino acids (Fig. 3). We found a serine-threonine-phenylalanine-lysine tetrad (STFK), which is characteristic of β-lactamase-active sites, at amino acid positions 86 to 89. A putative ATG initiation codon found at positions 303 to 305 was preceded by a ribosome-binding site (AGGATA), and the open reading frame was terminated by a TAA codon at positions 1218 to 1220. The only probable promoter found was between positions 27 and 57, which was 246 bp before the ATG initiation codon. The −10 sequence TATAAG began at position 52, and the −35 sequence TTCACA began at position 27. Following the stop codon, we noted an AT-rich region. At positions 1255 to 1299 (35 bp after the TAA codon) there is a possible hairpin-loop RNA secondary structure with a 20-bp stem and a 5-bp loop for a ΔG of −23.20 kcal/mol. The entire 1,775-bp sequence showed a G+C content of 40.79%, and the 915-bp structural gene showed a G+C content of 43.14%. The N-terminal amino acid sequence had a hydrophobic region that is known to be a common β-lactamase secretion signal. Taking into account that ROB-1 has the same length as the *Bacillus licheniformis* β-lactamase, we arbitrarily chose a signal peptide of 33 amino acids terminating at alanine (position 33). The mature protein would have an STFK-active site tetrad at positions 53 to 56 and an estimated molecular weight of 30,424.

Homology with other β-lactamases. The nucleotide sequence of the ROB-1 gene showed low homology with other

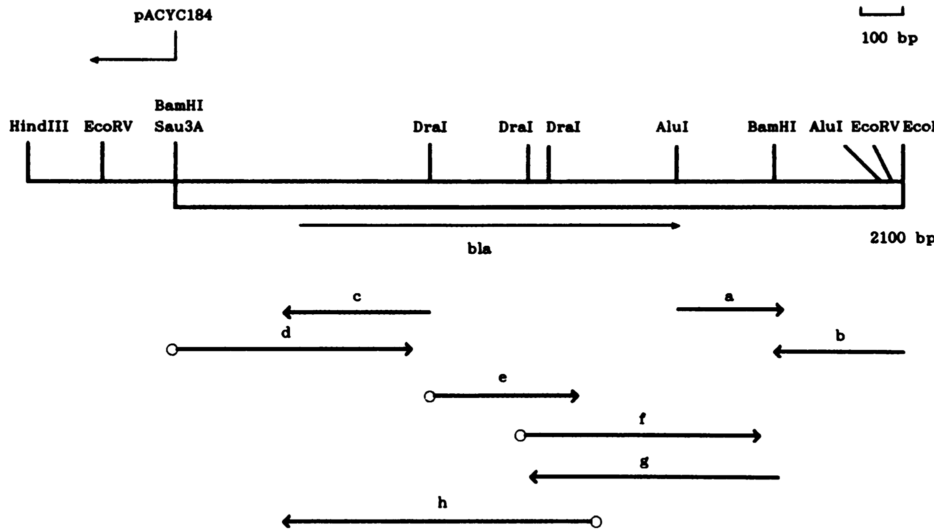

FIG. 2. Sequencing strategy used for the ROB-1 *bla* gene. The *Hin*dIII-*Eco*RI fragment of pMON401 is shown. Symbols: thin line, the vector; open box line, the insert; arrows, direction of nucleotide sequences analyzed; ○, oligonucleotide primers. Arrows a and b indicate sequences done in M13mp19; arrow c indicates those done in pTZ18R; and arrows d through h represent those done in pBGS18⁺ and in pBGS19⁺.

```
        ↓ BamHl/Sau3A            -35                               -10
   1    GGATCAGAGTAATAATTTCTGATGTTTTCACATAAATCACCCCCTTAGCACTATAAGGCTAAGCGACTAGGCTCACACTATGAACGGTAGTGCGAGCCGC    100

 101    CGCCATAGTGCGAAAGTGATTTTAACACATAACCGCCTAACAAGGCGTTATGTTTTATTCGGGGCTAAATTGCTAGACTTTTGCGCTTAAATTCGCCAAA    200
                                                                                                   RBS
 201    ATCTGTTTTTTTGTTCTACAAACAAATTATCCGAATTTCCGCGCAATGTACGTTTCAGGCTGCCTGAAAAGAAATTTATTTAGTAAAATCAAAGGATAAT    300

 301    TTATGTTAAATAAGTTAAAAATCGGCACATTATTATTGCTGACATTAACGGCTTGTTCGCCCAATTCTGTTCATTCGGTAACGTCTAATCCGCAGCCTGC    400
          M  L  N  K  L  K  I  G  T  L  L  L  L  T  L  T  A  C  S  P  N  S  V  H  S  V  T  S  N  P  Q  P  A

 401    TAGTGCGCCTGTGCAACAATCAGCCACACAAGCCACCTTTCAACAGACTTTGGCGAATTTGGAACAGCAGTATCAAGCCCGCATTGGCGTTTATGTATGG    500
          S  A  P  V  Q  Q  S  A  T  Q  A  T  F  Q  Q  T  L  A  N  L  E  Q  Q  Y  Q  A  R  I  G  V  Y  V  W
                                                                                                     | →
 501    GATACAGAAACGGGACATTCTTTGTCTTATCGTGCAGATGAACGCTTTGCTTATGCGTCCACTTTCAAGGCGTTGTTGGCTGGGGCGGTGTTGCAATCGC    600
        D  T  E  T  G  H  S  L  S  Y  R  A  D  E  R  F  A  Y  A |S  T  F  K| A  L  L  A  G  A  V  L  Q  S  L
                       ↓ DraI
 601    TGCCTGAAAAAGATTTAAATCGTACCATTTCATATAGCCAAAAAGATTTGGTTAGTTATTCTCCCGAAACCCAAAAATACGTTGGCAAAGGCATGACGAT    700
          P  E  K  D  L  N  R  T  I  S  Y  S  Q  K  D  L  V  S  Y  S  P  E  T  Q  K  Y  V  G  K  G  M  T  I

 701    TGCCCAATTATGTGAAGCAGCCGTGCGGTTTAGCGACAACAGCGCGACCAATTTGCTGCTCAAAGAATTGGGTGGCGTGGAACAATATCAACGTATTTTG    800
          A  Q  L  C  E  A  A  V  R  F  S  D  N  S  A  T  N  L  L  L  K  E  L  G  G  V  E  Q  Y  Q  R  I  L
                 2 →                                       ↓ DraI
 801    CGACAATTAGGCGATAACGTAACCCATACCAATCGGCTAGAACCCGATTTAAATCAAGCCAAACCCAACGATATTCGTGATACGAGTACACCCAAACAAA    900
        R  Q  L  G  D  N  V  T  H  T  N  R  L  E  P  D  L  N  Q  A  K  P  N  D  I  R  D  T  S  T  P  K  Q  M
                  ↓ DraI
 901    TGGCGATGAATTTAAATGCGTATTTATTGGGCAACACATTAACCGAATCGCAAAAAACGATTTTGTGGAATTGGTTGGACAATAACGCAACAGGCAATCC    1000
          A  M  N  L  N  A  Y  L  L  G  N  T  L  T  E  S  Q  K  T  I  L  W  N  W  L  D  N  N  A  T  G  N  P
                                          ← 3
1001    ATTGATTCGCGCTGCTACGCCAACATCGTGGAAAGTGTACGATAAAAGCGGGGCGGGTAAATATGGTGTACGCAATGATATTGCGGTGGTTCGCATACCA    1100
          L  I  R  A  A  T  P  T  S  W  K  V  Y  D  K  S  G  A  G  K  Y  G  V  R  N  D  I  A  V  V  R  I  P

1101    AATCGCAAACCGATTGTGATGGCAATCATGAGTACGCAATTTACCGAAGAAGCCAAATTCAACAATAAATTAGTAGAAGATGCAGCAAAGCAAGTATTTC    1200
        N  R  K  P  I  V  M  A  I  M  S  T  Q  F  T  E  E  A  K  F  N  N  K  L  V  E  D  A  A  K  Q  V  F  H
                                                                                     TRM
1201    ATACTTTACAGCTCAACTAACAAATTCATTTTGTTAGAAAAAGTGAAAATTTAGGACTGAAAAAGCCGTTGAAACTTGCTTTCAATGGCTTTTTTAGTCT    1300
          T  L  Q  L  N  *

1301    AACAACCGCAGGGCGTTAGCAGAAAGGTGCACGAATTTTCATAAAATTACACGGTAAGAAAGGTGTATGGATTTTCACAAAGGTGTACAGATTTTAATAA    1400
                                                      ↓ BamHI
1401    AATACACCTTAAAGCCCTTTAATACCCAATGCTTTAGAAAAGAAAAAGGGATCCCAGGGATTTCAGTTGTAGAACAAGGTCGCGGTGCAAGTTCTGGATA    1500

1501    TTCTATTCGTGGTATGGACAGAAATAGAGTTGCTTTATTAGTAGATGGTTTACCTCAAACGCAATCTTATGTAGTGCAAAGCCCTTTAGTTGCTCGTTCA    1600

1601    GGATATTCTGGCACTGGTGCAATTAATGAAATTGAATATGAAAATGTAAAGGCCGTCGAAATAAGCAAGGGGGGGAGTTCTTCTGATGTATGGTAATGGA    1700
                                                 ↓ EcoRV                     ↓ EcoRI
1701    GCACTAGCTGGTTCTGTAACATTTCAAAGCAAATCAGCAGCCGATATCTTAGAAGGAGACAAATCATGGGGAATT  1775
```

FIG. 3. Nucleotide sequence of the ROB-1 β-lactamase gene. The proposed ribosome-binding site is underlined and identified by −10 and −35. The most probable hairpin loop is depicted as follows: the stem is underlined, the loop is overlined, and the extremities are indicated by arrows. The deduced amino acid sequence is designated by the one-letter code, and the active site STFK is delimited by a box. Restriction sites are indicated by arrows. Oligonucleotides used in the sequencing strategy (Fig. 2) are overlined and indicated by numbers 1 through 3. The stop codon is represented by an asterisk.

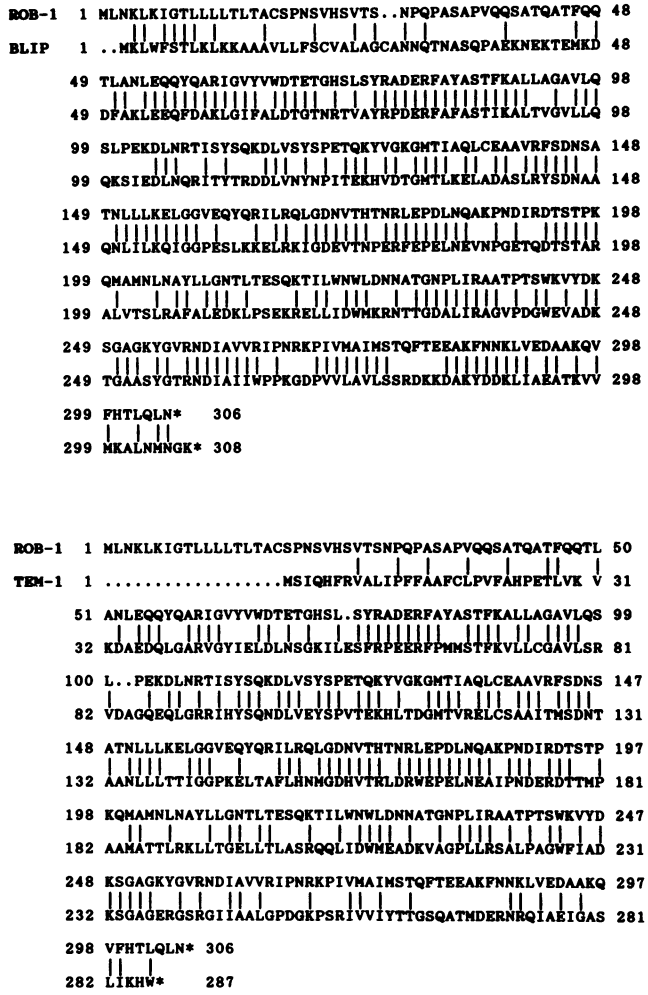

FIG. 4. Similarity of ROB-1 with *B. licheniformis* β-lactamase (BLIP) and TEM-1. The degree of similarity between ROB-1 and the β-lactamase from *B. licheniformis* is 63.48%, and that between ROB-1 and TEM-1 is 55.63%. Stop codons are represented by asterisks.

known class A, B, C, and D β-lactamases. However, comparisons of amino acid sequences showed interesting features. The deduced ROB-1 amino acid sequence was compared with β-lactamases by using the Gap program from the University of Wisconsin and the Align program of Dayhoff (13). The Gap program, which is based on a table of relatedness between amino acids, aligns identical and similar amino acids (pairing values, <0 and ≥ 1.5). Similarity with class B, C, and D β-lactamases was low (below 40%), while comparisons with class A enzymes gave higher values. The highest similarity for ROB-1 was found with β-lactamase I of *Bacillus cereus* (39) (63.48%) and β-lactamase of *B. licheniformis* (28) (63.48%). The lowest similarity was 48.28% with the β-lactamase of *Rhodopseudomonas capsulata* (9). In Fig. 4, we present the alignments obtained between ROB-1 and the class A β-lactamase TEM-1 and *B. licheniformis*. From these results, it is apparent that ROB-1 is a class A β-lactamase, but in contrast to what we expected, the highest similarity observed was not with TEM-1. Indeed, similarity values between ROB-1 and β-lactamases of gram-positive bacteria were consistently higher.

To assess the relatedness between ROB-1 and other β-lactamases, we found it essential to compare these proteins by using the mutation data matrix of Dayhoff (13) and Align software, which can detect distance relationships in proteins. Alignment scores (in standard deviation units) with class B, C, and D enzymes and ROB-1 were very low (<3.35), while alignment values with class A enzymes were high (>25). The highest value obtained (50.3) was with the β-lactamase of *B. licheniformis*, and the lowest value obtained (24.7) was with PSE-4. TEM-1 had a score of 27.7. ROB-1 had higher scores with β-lactamases from gram-positive bacteria (33.7 to 50.3) than it did with β-lactamase from gram-negative bacteria (24.7 to 27.7).

Taking into account previous results, we aligned class A β-lactamases (Fig. 5). The ROB-1 peptide sequence was fitted in the alignment by using no additional padding and contained the seven conserved boxes typical of penicillin-interactive proteins. These β-lactamases can be classified into two distinct groups reflecting computer comparisons between each β-lactamase, as shown in Fig. 5A and B. These enzymes are presumably in two evolutionarily related families, when taking into account scores of the Align program. The two groups represent β-lactamases from gram-positive (Fig. 5A) and gram-negative (Fig. 5B) bacteria, except for ROB-1. Thus, we classified ROB-1 in the first group because similarity and distance relationship values obtained when ROB-1 was compared with all class A β-lactamases showed that it is more closely related to the enzymes from gram-positive bacteria.

DISCUSSION

We sequenced the complete ROB-1 structural gene, including 302 bp before and 555 bp after the structural gene. The putative promoter, ribosome-binding site, and terminator sequences suggest that the gene that we analyzed is complete. It could be possible that the ROB-1 structural gene is driven by the *tet* promoter in pMON401 since the first cloning step was in the *Bam*HI site of pACYC184 (30). However, the subcloning of a *Hin*dIII-*Bam*HI fragment in pBGS18$^+$ and pBGS19$^+$ gave ampicillin resistance. Since the *Hin*dIII site is into the *tet* promoter, one would expect that the ROB-1 gene is controlled by its own promoter in *E. coli*.

One intriguing question is what is the origin of the ROB-1 β-lactamase gene found in *H. influenzae*? The G+C content of 40.79% for the entire sequence and 43.14% for the ROB-1 structural gene is typical for *H. influenzae* (37 to 44%) and for members of the family *Pasteurellaceae* (38 to 47%) (24). Codon usage in the ROB-1 gene is similar to that in the two sequenced genes (polysaccharide export protein and outer membrane protein p1) of *H. influenzae* (20, 27).

A search for amino acid sequence similarity showed that ROB-1 is a class A enzyme related to β-lactamases of gram-positive bacteria. This was confirmed by measuring the evolutionary distance by using a mutation matrix. The preliminary data showed that ROB-1 is highly related to class A β-lactamases but is not significantly related to enzymes in classes B, C, and D. However, we suggest that it is possible to divide class A enzymes into two groups, which is in agreement with the classification based on their bacterial origin and biochemistry (7, 8).

The first group includes β-lactamases from gram-positive bacteria such as *B. cereus*, *Streptomyces cacaoi*, *B. licheniformis*, *Staphylococcus aureus*, and *H. influenzae* (ROB-1). β-Lactamases of *Streptomyces aureofaciens* and *Streptomyces albus* are related but presumably divergent in their own

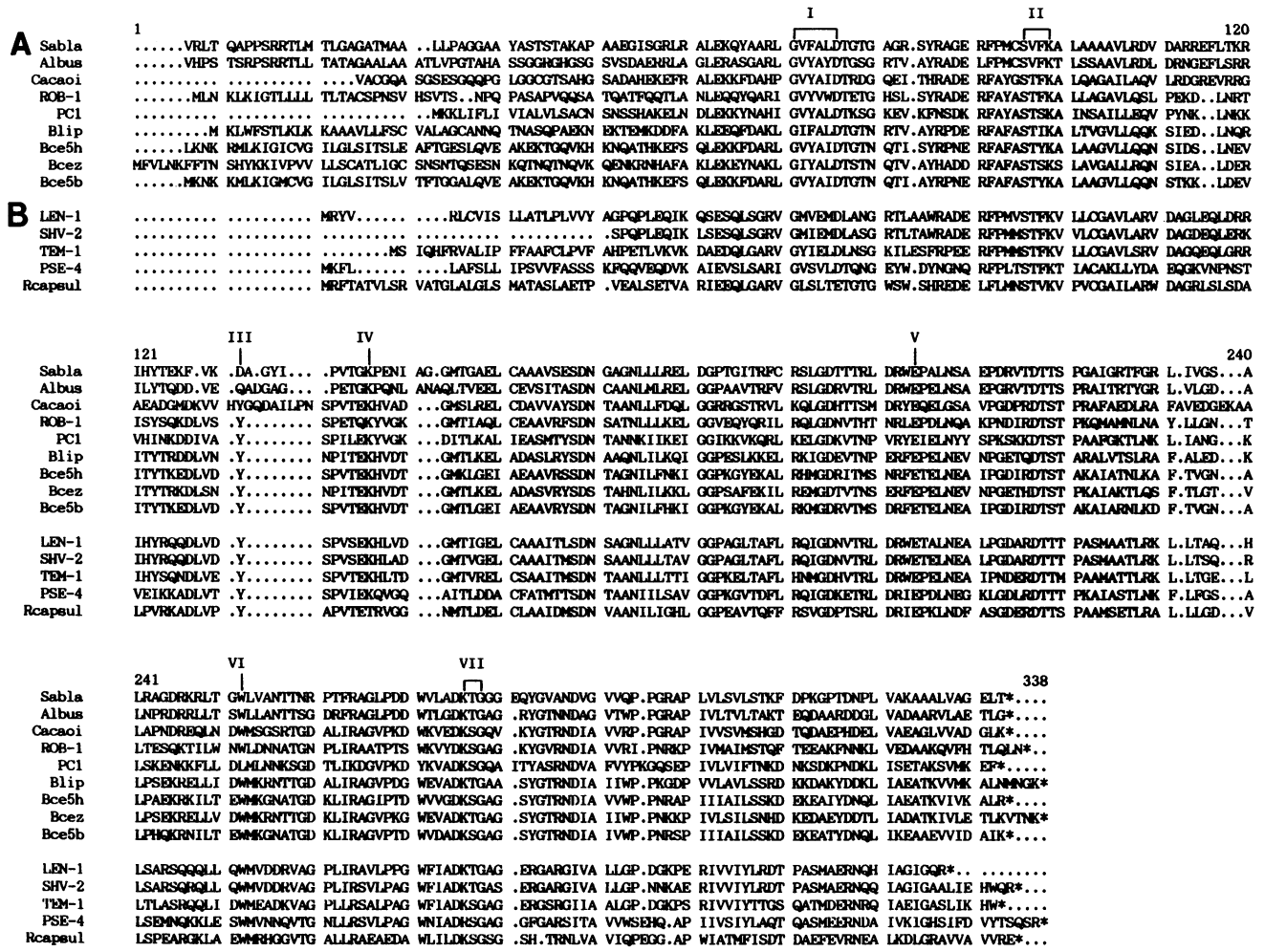

FIG. 5. Alignment of the ROB-1 amino acid sequence with other class A β-lactamases. Gaps were introduced by hand, taking into account the highest similarity. Conserved boxes are numbered I to VII. Abbreviations: Sabla, β-lactamase of *Streptomyces aureofaciens* (J. P. Reynes, et al. European Molecular Biology Laboratory data library, accession number X13597); Albus, β-lactamase of *Streptomyces albus* G (14); Cacaoi, chromosomal β-lactamase from *Streptomyces cacaoi* KCC-SO352 (21); ROB-1, β-lactamase from *H. influenzae*; PC1, β-lactamase from *Staphyloccocus aureus* PC1 (10); Blip, β-lactamase from *B. licheniformis* 749/c (28); Bce5h, β-lactamase I from *B. cereus* 569/H (34); Bcez, β-lactamase III from *B. cereus* 569/H (18); Bce5b, β-lactamase I from *B. cereus* 5/B (39); LEN-1, β-lactamase from *Klebsiella pneumoniae* LEN-1 (2); SHV-2, β-lactamase from *E. coli* A2302 (4); TEM-1, β-lactamase from *Salmonella paratyphi* B (36); PSE-4, β-lactamase from *Pseudomonas aeruginosa* strain Dalgleish (5); Rcapsul, β-lactamase from *R. capsulata* sp108 (9). Stop codons are represented by asterisks.

subgroup. We also noted that ROB-1 has the same peptide length as those of other β-lactamases in group I. Interestingly, all β-lactamases of group I, except for ROB-1, are in group 2a in the biochemical classification of Bush (7). In group II, plasmid-mediated β-lactamases from gram-negative bacteria include enzymes that have been identified as broad spectrum and carbenicillinase (groups 2b and 2c) (8).

The alignment of class A β-lactamases (Fig. 5) showed the seven boxes that are conserved in proteins that interact with β-lactam compounds (19). The ROB-1-active site is assumed to be an STFK (box II) tetrad and KSG triad (box VII), as for TEM-1. In addition to these boxes, we found additional amino acid residues conserved in all class A β-lactamases. It is not known (but is strongly suggestive) whether conserved residues may be implicated in structure and enzymatic activities typical of class A β-lactamases. This hypothesis needs to be confirmed by site-specific mutagenesis. Finally, analysis of the ROB-1 β-lactamase at the sequence and evolutionary levels suggests that it did not necessarily originate in *H. influenzae* and could have been acquired by lateral transfer between bacterial species.

ACKNOWLEDGMENTS

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